NOTES ON THE PREPARATION OF NATURAL HISTORY SPECIMENS FOR SCANNING ELECTRON MICROSCOPY

By R. H. HARRIS, B. S. MARTIN, & C. G. OGDEN

CONTENTS

INTRODUCTION

In the course of assisting members of the five Departments of the British Museum (Natural History) in the preparation of their materials for examination by scanning electron microscopy, the authors have gained considerable experience in dealing with a variety of specimens requiring different treatments. Although it would be impracticable to describe all the methods used on such diverse specimens, an attempt is made here to present an account of the basic techniques used in these preparations.

As most biological materials are non-conductors, it is necessary to apply a conductive covering over the entire surface of the object before examination. The biological specimens referred to here can be divided into two groups, loosely described as being either soft or hard. The soft specimens, whether fresh, fixed or preserved, are usually freeze-dried to maintain their natural appearance, whilst the hard materials require the minimum of treatment prior to coating. Regardless of the number of stages through which the material is passed, it is essential to take the utmost care with the preparations to ensure that the specimens maintain their shape and structure, and to guard against the introduction of artifacts.

The importance attached to preparation is perhaps more easily explained by considering the example in which only a few specimens are available. If, after processing, it is seen that extraneous material is adhering to the surface, it is possible that it will be obscuring essential detail. Under these circumstances valuable information could be lost, because the removal of the conductive layer from biological specimens is difficult, and this prevents the possibility of their being cleaned and recoated.

No attempt is made to include reference to the numerous publications containing details relating to the preparation of specific tissues or specimens, but reference is made to some recent general accounts.

FIXATION

In certain circumstances it is not convenient to treat fresh material by direct freeze drying. This applies to specimens like blood-cells which are difficult to separate from the surrounding plasma, and to specimens that are difficult to maintain outside their natural environment. In these cases the use of a chemical fixative prior to freeze drying is recommended.

The purpose of using a fixative is to preserve the detail of the material faithfully at the time of fixation. Although this would appear to apply only to transmission electron microscopy, in which detail of cellular structure is examined down to the molecular level, it applies equally to scanning electron microscopy, even though the fine structure is confined mainly to the external surface.

In general, the conventional transmission electron microscopy fixatives, glutaraldehyde and osmium tetroxide both suitably buffered, have proved to be satisfactory. There are, however, some exceptions, for example, human blood-cells when treated for one hour in neutral Kaeserling I fixative produce good results (Pl. 1, fig. A). Some success has been achieved with Parduczs' fixative, as recommended by Small & Marszalek (1969), but the subsequent washing required to ensure the removal of the introduced salts, makes this method tedious. In some circumstances it has been found necessary to narcotize the specimens prior to fixation. Certain oligochaetes and platyhelminthes not only contract violently if plunged directly into fixatives, but also secrete mucous which is often so finely dispersed (Pl. 1, fig. B) that it is only detected on examination at magnifications outside the range of the optical microscope. The conventional narcotic substances, menthol, ethyl urethane, magnesium chloride, chloral hydrate and alcohol, are suitable for most specimens. Nevertheless, marine zooplankton produce better results when treated with a solution of 100 mg l^{-1} tricaine methanesulphate. The specimens must be washed thoroughly after using fixatives to ensure that no salts are deposited in the subsequent freeze drying.

FREEZE DRYING

The tissue to be examined is carefully washed in several changes of distilled water, with a final rinse in triple glass distilled water. Normal and double distilled water leave a considerable deposit of dissolved salts after sublimation. Each specimen is treated separately in a Durham's fermentation tube, sufficient water being retained with delicate tissues to support them in a life-like position, whilst all the water is drained from firm tissues.

The specimens are rapidly frozen by spraying them for a few seconds with Polar Spray (100% dichlorodifluoromethane in an aerosol container). They are immediately placed in the specimen-chamber of an Edwards Ef2 freeze dryer, previously pumped down to a working pressure of 50 μm Hg (0·05 Torr), with a specimen

temperature of −15°C and condenser temperature of −40°C. The apparatus is activated and sublimation is carried out for twelve hours. When the specimens are taken from the chamber they are placed into a small desiccator containing silica gel for at least half an hour. This allows any condensation, formed on removal from the cool chamber, to be adsorbed.

In some cases the specimens, particularly preserved materials, have an excess of debris adhering to them, but it is possible to clean these by using liquid-nitrogen. Care must be taken in handling liquified gases, and it is essential that all equipment is cooled prior to use. The material to be treated must also be pre-cooled to prevent the formation of a 'Leidenfrost envelope', which could cause considerable damage to cell-membranes.

The specimens to be treated with liquid-nitrogen, after washing and draining, are cooled for a few seconds using a Polar Spray. Then, using a pair of pre-cooled forceps, the tubes are carefully dipped beneath the surface of liquid-nitrogen. A small thermos flask, previously cooled to −20°C for several hours, provides a suitable liquid-nitrogen container. The tubes are removed from the liquid-nitrogen and immediately placed in the specimen chamber of a freeze dryer and sublimated as before. The small amount of liquid-nitrogen remaining in the tubes boils rapidly, and this effectively removes the surface contamination from the specimen.

ADHESIVES AND MOUNTANTS

The Cambridge 'Stereoscan' specimen stub is a circular, aluminium platform, 13 mm in diameter, with grooved edges for handling. The critical dimensions are those of the shank which has a diameter of 3·2 mm ($\frac{1}{8}$″) and a length of 8 mm. Alternative specimen supports can be used, for example, $\frac{1}{8}$″, flat-headed, aluminium rivets, but these are difficult to handle and the standard stub is preferred.

A large number of adhesives are used to attach specimens to the stubs, but only a few of these appear to be suitable. An investigation by Muir & Rampley (1969), together with observations made in this Museum, suggest that most adhesives are easily damaged by the electron beam. This damage appears to the viewer as 'squares' or 'lines' of sharply different contrast compared with the remaining areas of the exposed adhesive surface. In addition, some are unstable and change their dimensions constantly in the high vacuum of the coating unit and the microscope specimen-chamber, possibly due to slow solvent evaporation. Specimens are prone to move under these conditions, making focusing difficult, and accurate recording impossible. Decomposition products are also produced which gradually contaminate the microscope column.

Three commercially available adhesives, 'Araldite', 'Silver Dag' and 'Silver Mounting Compound', are stable in the scanning electron microscope. 'Araldite' is a good adhesive, but has the disadvantage of being an electrical insulator, whereas 'Silver Dag' is electrically conductive, but has inferior adhesive properties. 'Silver Mounting Compound' is a general term for a series of epoxy resin/colloidal silver mixtures, produced by Johnson-Matthey Ltd., which combine the best properties of both materials.

There are occasions when the adhesives recommended above are not convenient to use. Some entomological specimens, for example, are more easily mounted on double-sided adhesive tape. This tape exhibits all the defects mentioned above and ages badly. This latter feature causes a breakdown of electrical continuity over the stub surface, which becomes apparent on re-examination after a short period of storage. Such adhesives are acceptable, provided that the disadvantages are borne in mind and a more suitable product is used whenever possible.

A point often overlooked when mounting specimens is the part played by the adhesive in the background of the anticipated micrographs. Double-sided tape (Pl. 1, fig. C) and 'Silver Dag' have an unpleasant and confusing appearance, whilst the stub itself is scored with machine marks and small pits. A clean, clear background is easily obtained by sticking a 10 mm diameter, circular, glass cover-slip on to the stub-surface with 'Silver Dag', to ensure that good contact is made with the coating material.

SPECIMEN COATING

All biological specimens are electrical insulators and it is necessary to coat them with a conductive layer, usually an evaporated metal, to provide an electrical path to earth. The absence of this layer causes an electrical charge to accumulate on the specimen with resulting loss of resolution (Pl. 1, fig. C), and the appearance of bright patches that obscure detail. This progresses until the whole image area becomes speckled with bright flashes or streaked in the direction of the scanning beam. The maximum resolution of the Stereoscan is 20 nm, and it is inadvisable to exceed this thickness with the coating material if an accurate record is to be obtained. In practice a coating thickness of between 10 and 15 nm is considered satisfactory for most specimens.

Aluminium, magnesium, tin, cadmium, molybdenum, gold, gold/palladium alloy, silver, copper, platinum, carbon and anti-static solutions have been tried as coating materials with varying degrees of success. Aluminium has a high secondary electron emission and probably gives the strongest signal image formation. It has, however, two disadvantages, both of which cause coating instability. Any change in specimen dimensions due to shrinkage or local heating effects are inclined to cause cracks that charge, and the rapid oxidation of the metal surface in air causes local areas of insulation. The advantage it has over gold, is that it can be easily removed (Sylvester-Bradley, 1969) by immersion in a freshly made solution of sodium hydroxide. The precious metals, due to their malleability and inherent stability, appear to give the most satisfactory results. Gold is preferred, because it is easy to handle, it does not form an alloy with the tungsten filaments from which it is evaporated and it is readily available in wire form. In addition, it is removable (Hansen, 1968) by using an alkaline solution of sodium cyanide, although this only applies to hard materials.

Some specimens, mainly entomological, have features whose origins are situated in recesses below the general surface level, for example, insect appendages and setae. In these cases, it is impossible for the evaporated metal to reach these innermost

regions and make a continuous conductive layer. This can be remedied by soaking the specimens in a solution of 0·5% Duron, in iso-butyl alcohol, for a period of between twelve and twenty-four hours. Duron is an anti-static solution, and was initially used by Sikorski, *et al.* (1967), as a direct coating preparation for certain polymers. After soaking, the specimens are removed from the solution, washed briefly with iso-butyl alcohol and allowed to dry. They are then coated in the normal manner using evaporated metal. It is necessary to take the utmost care in the washing stage of this sequence, to ensure that the anti-static solution is washed from the exposed body surface, but not out of the recesses.

The coating metal is evaporated, in vacuo, from 'V'-shaped, tungsten filaments. During evaporation the specimen stubs are rotated by means of an apparatus (Plate 2, figs A & B) developed from that described by Boult & Brabazon (1968). Four filaments are used to ensure that, in conjunction with the changing attitude of the stubs during rotation, the metal is coated evenly over the specimen through a complete range of angles from glancing (Plate 2, fig. B) to normal incidence. It cannot be too strongly emphasized that coating failures frequently occur if the lower filaments, which provide coating at very low angles to the stub-surface, are badly positioned or omitted. The stub-platten (Plate 2, fig. B) is rotated at about one revolution per second, and each stub revolves on its own axis between five and six times per second. As the total evaporation time is between ten and fifteen seconds an even coating is ensured.

The thickness of the metal coat on any given specimen is difficult to determine. However, if one considers the specimen to be flat, it is possible to estimate the mass of metal required for a known coating thickness. It is considered that metal deposited from filaments 1 & 2 (Plate 2, fig. A) will not contribute significantly to the coating on that part of the specimen normal to filaments 3 & 4, and vice versa. Making this assumption, the metal required for each filament can be calculated as follows:

$$m = \frac{8}{3} \pi . t . R^2 . p$$

where, m = mass of metal per filament (g); t = coating thickness required (cm); R = average filament to specimen stub distance (cm) and p = density of metal used ($g.cm^{-3}$).

Alternatively, this may be expressed in terms of wire length as:

$$l = \frac{8 . R^2 . t}{3 . r^2}$$

where, r = radius of wire (cm) and l = length of wire (cm).

In practice, using 36 swg (0·0193 cm diameter) gold wire, each of the four filaments carries 6 cm of metal and is positioned at a distance of 12 cm from any given stub. This gives a coating thickness of 15 nm, which has been confirmed by measurements obtained using a quartz crystal thickness monitor. Evaporation takes place in a vacuum coating unit at a pressure of 10^{-4} Torr, each filament being heated to allow evaporation to be completed within ten to fifteen seconds.

The success of this coating procedure depends on the adequate physical contact between the specimen and the stub. Large specimens such as pieces of rock and

marine shells often lack this contact, as do insects which are frequently attached to the stub solely by means of their appendages or other fine structures. In these cases filling the gap between the lower surface and the stub with 'Silver Dag' improves the electrical contact. Alternatively, a second coating from the lower pair of filaments often solves the problem. It is thought that the surface of some specimens tends to repel the metal coat, due to a waxy or oily covering. In these circumstances, the only alternative appears to be the evaporation of a larger quantity of metal in the hope that sufficient will adhere. The surface tension of molten gold is such that longer lengths of wire cannot be wound on a normal 'V' filament, but this is overcome by making a double 'V' filament.

The coating on the surface of chitinous materials, such as hair and insect cuticle, is often seen to be crazed (Plate 1, fig. D). No means of eliminating this effect has yet been found, but prolonged air drying prior to mounting appears to reduce it to an acceptable level.

REFERENCES

Boult, E. H., & Brabazon, E. J. 1968. A rotation device for use in metallizing nonconducting scanning electron microscope specimens. *J. Sci. Instrum.* (*J. Phys. E.*) **1** (Ser. 2) : 565–566.

Hansen, H. J. 1968. A technique for removing gold from plated calcareous microfossils. *Micropaleontology* **14** : 499–500.

Muir, M. D. & Rampley, D. N. 1969. The effect of the electron beam on various mounting and coating media in scanning electron microscopy. *J. Microscop.* **90** : 145–150.

Sikorski, J., Nott, J. A., Moss, J. S., & Buckley, T. 1967. A new preparation technique for examination of polymers in the scanning electron microscope. *Proc. R. microsc. Soc.* **2** : 431–432.

Small, E. B., & Marszalek, D. S. 1969. Scanning electron microscopy of fixed, frozen, and dried Protozoa. *Science* **163** : 1064–1065.

Sylvester-Bradley, P. C. 1969. Aluminium coating in scanning electron microscopy. *Micropaleontology* **15** : 366.

R. H. Harris
B. S. Martin
C. G. Ogden
Electron Microscope Unit
British Museum (Natural History)
Cromwell Road
London SW7 5BD

PLATE 1

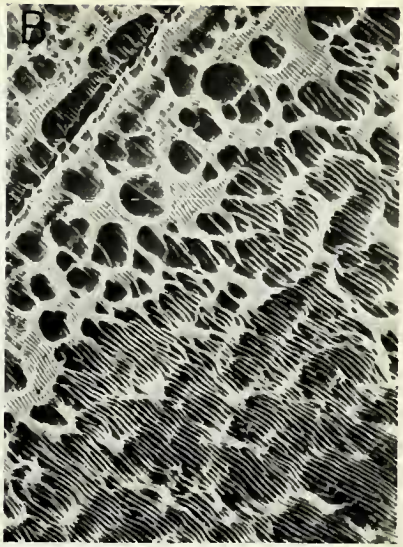
FIG. A. Human erythrocytes. ×10,000.

FIG. B. Cuticle of a nematode (*Mermis nigrescens*) showing mucus pattern. ×1,420.

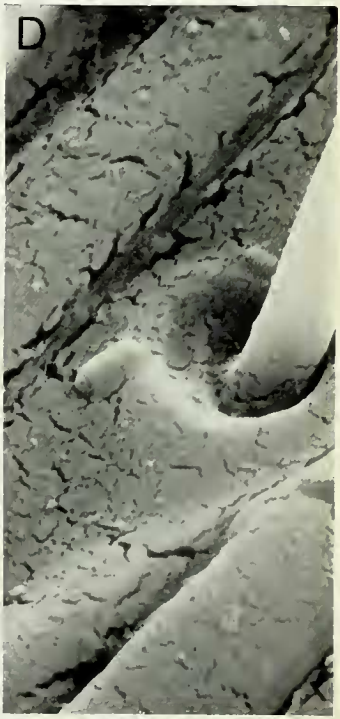
FIG. C. Two mites (*Dermanyssus gallinae*), the upper of the two shows typical charging. The background pattern is due to double-sided adhesive tape. ×75.

FIG. D. The cuticle of a mite, showing the crazing effect associated with chitinous surfaces. ×75,000.


A


B


C


D